Box Seq 103 CO

Atty Docket: OXVIG=1A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Application Division
Claus OXVIG	)	**ATTN: BOX MISSING PARTS**
Serial No.: 09/983,025	)	Washington, D.C.
Filing Date: October 22, 2001	)	Confirmation No. 7756
For: PREGNANCY-ASSOCIATED PLASMA PROTEIN-A2	)	January 14, 2002

OIPE JC46 JAN 14 2002 PATENT & TRADEMARK OFFICE

LATE SUBMISSION OF FILING FEE AND/OR DECLARATION

HON. COMMISSIONER OF PATENTS
Washington, D.C. 20231
Sir:

The present communication is in response to the "NOTICE TO FILE MISSING PARTS OF APPLICATION..." dated November 14, 2001.

[XX] Attached hereto is an executed oath or declaration in compliance with 37 C.F.R. 1.63, identifying the present application by title and priority information.
[XX] Substitute Drawings in compliance with 37 CFR 1.84.
[XX] Applicant claims small entity status. See 37 CFR 1.27.
[ ] A Communication.
[XX] Preliminary Amendment.
[XX] Sequence Listing (hardcopy), and computer-readable form of Sequence Listing.
[XX] A certified copy of Danish priority document no. PA 2000 01571, filed October 20, 2001, is also attached. Priority is claimed.
[ ] An Information Disclosure Statement with 08A-1449 and ___ references is also attached.

The following fee is calculated below:

	(Col. 1)	(Col. 2)		SMALL ENTITY			OTHER THAN SMALL ENTITY	
FOR:	NO FILED	NO EXTRA		RATE	FEE	OR	RATE	FEE
BASIC FEE					$370 00	OR		$740.00
TOTAL CLAIMS	64 - 20	44		X 9 =	$396 00		X 18 =	$
INDEP. CLAIMS	4 - 3	1		X 42 =	$ 42.00		X 84 =	$
[ ] MULTIPLE DEPENDENT CLAIMS PRESENTED				+ 135 =	$		+ 270 =	$
If the difference in Col 1 is less than 0, enter "0" in Col 2				TOTAL	$808.00		TOTAL	$

[ ] Surcharge for late filing of English translation $ 130.00
[XX] Late filing of Declaration surcharge in the amount of:
Small Entity [XX] $65.00 Other than Small Entity [ ] $130.00
[ ] It is hereby petitioned for an extension of time in accordance with 37 C.F.R. 1.136(a). The appropriate fee required by 37 C.F.R. 1.17 is calculated as shown below:

Small Entity Response Filed Within		Other Than Small Entity Response Filed Within	
[ ] First	$ 55.00	[ ] First	$ 110.00
[ ] Second	$ 200.00	[ ] Second	$ 440.00
[ ] Third	$ 460.00	[ ] Third	$ 920.00
[ ] Fourth	$ 720.00	[ ] Fourth	$1,440.00
Month After Time Period Set		Month After Time Period Set	

[XX] Conditional Petition for Extension of Time:
If any extension of time for a response is required, applicant requests that this be considered a petition therefor.
[ ] Check No. _____ in the amount of $_____ is enclosed to cover the above fees.
[XX] Credit Card Payment Form, PTO-2038, authorizing payment in the amount of $873.00 is enclosed to cover the fees.
[XX] The Commissioner is hereby authorized and requested to charge any additional fees which may be required in connection with this application or credit any overpayment to Deposit Account No. 02-4035. This authorization and request is not limited to payment of all fees associated with this communication, including any Extension of Time fee, not covered by check or specific authorization, but is also intended to include all fees for the presentation of extra claims under 37 CFR 1.16 and all patent processing fees under 37 CFR 1.17 throughout the prosecution of the case. This blanket authorization does not include patent issue fees under 37 CFR 1.18.

BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant(s)

By: S. Neimark
~~Ivan P. Cooper~~
Registration No. ~~20,005~~ 20520

IPC:sfg

UNITED STATES PATENT AND TRADEMARK OFFICE

COMMISSIONER FOR PATENTS
UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, D.C. 20231
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APPLICATION NUMBER	FILING/RECEIPT DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
09/983,025	10/25/2001	Claus Oxvig	OXVIG=1A

CONFIRMATION NO. 7756

FORMALITIES LETTER

OC000000007068153

BROWDY AND NEIMARK, P.L.L.C.
624 Ninth Street, N.W.
Washington, DC 20001

Date Mailed: 11/14/2001

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

01/17/2002 HNOOR1 00000074 09983025

FILED UNDER 37 CFR 1.53(b)

01 FC:201 370.00 OP
02 FC:205 65.00 OP
03 FC:202 42.00 OP
04 FC:203 396.00 OP

Filing Date Granted

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

MSP=14JA2002
DRG=14JA2002
SEQ=14JA2002

- The statutory basic filing fee is missing.
  Applicant must submit $ **370** *to complete the basic filing fee for a small entity.*
- Total additional claim fee(s) for this application is $1421.
  - **$1197** for **133** total claims over 20.
  - **$84** for **2** independent claims over 3 .
  - **$140** for multiple dependent claim surcharge.
- The oath or declaration is missing.
  A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.
- To avoid abandonment, a late filing fee or oath or declaration surcharge as set forth in 37 CFR 1.16(l) of $65 for a small entity in compliance with 37 CFR 1.27, must be submitted with the missing items identified in this letter.
- **The balance due by applicant is $ 1856.**

The application is informal since it does not comply with the regulations for the reason(s) indicated below.

The required item(s) identified below must be timely submitted to avoid abandonment:

- Substitute drawings in compliance with 37 CFR 1.84 because:
  - drawing sheets do not have the appropriate margin(s) (see 37 CFR 1.84(g)). Each sheet must include a top margin of at least 2.5 cm. (1 inch), a left side margin of at least 2.5 cm. (1 inch), a right side margin of at least 1.5 cm. (5/8 inch), and a bottom margin of at least 1.0 cm. (3/8 inch);

- This application does not contain a statement that the content of the sequence listing information recorded

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	**Conf. No.: 7756**
	)	
OXVIG et al	)	Examiner:
	)	
Appln. No.: 09/983,025	)	Washington, D.C.
	)	
Filed: October 22, 2001	)	January 11, 2002
	)	
For: PREGNANCY-ASSOCIATED PLASMA PROTEIN-A2 (PAPP-A2)	)	Atty.Docket: OXVIG=1A

RESPONSE TO "SEQUENCE LISTING" REQUIREMENT

Honorable Commissioner of Patents
Washington, D.C. 20231

Sir:

In response to the Notice to Comply included in the Notice to File Missing Parts, mailed November 14, 2001, please amend the application as follows:

IN THE SPECIFICATION

Please amend the paragraph beginning at line 31 of page 9 with the following rewritten paragraph:

--Homology of PAPP-A2 with PAPP-A is evident upon alignment of the two amino acid sequences as shown in Figure 3. PAPP-A2 and PAPP-A share approximately 45% of their amino acid residues. Sequence motifs known to be important for the function of PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8; Lawrence et al., 1999, Proc Natl Acad Sci U S A 96, 3149-53; Overgaard et al., 2000, J Biol Chem) are also found

in PAPP-A2. Principally, PAPP-A2 contains an elongated zinc binding motif (HEXXHXXGXXH (SEQ ID NO:3), amino acids shown by one letter code) at position 733-743 (Figure 2). This motif and a structurally important methionine residue, are strictly conserved within the metzincins, a superfamily of zinc peptidases (Bode et al., 1993, FEBS Lett 331, 134-40; Stocker et al., 1995, Protein Sci 4, 823-40).--

Please amend the paragraph beginning at line 31 of page 50 with the following rewritten paragraph:

--Figure 1 shows the cDNA sequence (in 5'→3' orientation) corresponding to the mRNA that encodes preproPAPP-A2. Only the coding part of the sequence and the terminal stop codon (*) is shown and is numbered 1-5376 of SEQ ID NO:1. The translated polypeptide sequence (SEQ ID NO:2) of preproPAPP-A2 is also shown. The signal peptide cleavage site was predicted using SignalP V2.0 to be after the alanine residue encoded by nt. 64-66 ((Nielsen et al., 1997, Protein Eng 10, 1-6), WWW prediction server is located at http://genome.cbs.dtu.dk/). The signal peptide of preproPAPP-A2 (nt. 1-66, 22 residues) is shown in bold. The nucleotide sequence of this figure represents nt. 1 to 5376 of SEQ ID NO:1. The protein sequence of this figure is illustrated as SEQ ID NO:2.--

Please amend the paragraph beginning at line 26 of page 51 with the following rewritten paragraph:

--Figure 3 shows the amino acid sequence of preproPAPP-A2 (SEQ ID NO:2) aligned with preproPAPP-A. The deduced amino acid sequence of preproPAPP-A2 (PA2) was aligned with the sequence of preproPAPP-A (PA) (SEQ ID NO:25) ((Haaning et al., 1996, Eur J Biochem 237, 159-63), AAC50543) using CLUSTAL W (Thompson et al., 1994, Nucleic Acids Res 22, 4673-80). Because the prepro-portion of PAPP-A did not show significant identity with the corresponding region of PAPP-A2, the alignment was manually adjusted to emphasize difference in length of pro-peptides. Arrows indicate the N-termini of the mature proteins as found earlier for PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8) (Glu-81), and here for PAPP-A2 (Ser-234). Putative signal peptides, strongly predicted using SignalP V2.0 (Nielsen et al., 1997, Protein Eng 10, 1-6) are shown with lower case letters. The pro-portion of PAPP-A2 contains one other candidate initiation codon corresponding to Met-168, but no signal peptide was predicted following this residue using SignalP. The sequence motifs of PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8) are also found in PAPP-A2: The catalytic zinc binding motif and residues of the putative Met-turn are underlined and bolded in both sequences. Lin-notch motifs (LNR1-3) and short consensus repeats (SCR-1-5) are boxed. Cysteine residus are

shaded. All cysteines of mature PAPP-A are also found in PAPP-A2. In addition, the secreted form of PAPP-A2 has four cysteine residues (Cys-343, Cys-533, Cys-618, and Cys-1268) with no counterpart in PAPP-A.--

Please amend the paragraph beginning at line 8 of page 53 with the following rewritten paragraph:

--Figure 7 shows the cDNA sequence of the PAPP-A2 mRNA coding region directly followed by the sequence of the 3'UTR. The sequence of the 3'UTR was obtained as detailed in Example 6.3. The first 5376 nucleotides of this sequence (nt. 1 - 5376) represents the coding sequence as illustrated in Figure 1 and SEQ ID NO:1 (nt. 1 - 5376). Nucleotides 5377 - 8527 of this sequence corresponds to the 3'UTR of the PAPP-A2 mRNA as illustrated in SEQ ID NO:1 (nt. 5377 - 8527).--

Please amend Table 1 at line 20 of page 55 with the following rewritten Table 1:

--TABLE 1. Locations of primers used for reverse transcription or PCR. The primers are listed in the order of their use.

NAME	SOURCE[a]	Nt. NUMBERS[b]	SEQUENCE[c]
RT-N-mid:	AL031290	10262-10281, (4770-4789)	GCTCACACACCACAGGAATG* (SEQ ID NO:4)
PR-mid5:	AL031734	141874-141894, (1947-1967)	GGCTGATGTGCGCAAGACCTG (SEQ ID NO:5)
PR-mid3:	AL031290	10208-10229, (4716-4737)	GCATTGTATCTTCAGGAGCTTG* (SEQ ID NO:6)
PR-N5:	AL031734	102606-102628, (-)	GAAGTTGACTTCTGGTTCTGTAG (SEQ ID NO:7)

PR-N3:	-	-, (2380-2400)	CCCTGGGAAGCGAGTGAAGCC* (SEQ ID NO:8)
RT-C:	AL031290	62982-63006, (-)	GCATTTCTTATAAGATCCTTCATGC* (SEQ ID NO:9)
PR-C5:	-	-, (4180-4201)	GACAGCTGTCCGTCATTGCTGC (SEQ ID NO:10)
PR-C3:	AL031290	62876-62897, (-)	CTTACTGCCTCTGAGGCAGTGG* (SEQ ID NO:11)

[a]Accession numbers of the relevant genomic clones are given. Primers PR-N3 and PR-C5 were located in the sequence connecting hom-N and hom-C, and are therefore not represented in the databases.

[b]Nucleotide numbers refer to the numbering of the sequences as reported in the file with the relevant accession number. In parentheses are given the corresponding numbers of SEQ ID NO:1 (Figure 1), except for primers PR-N5, RT-C and PR-C3, not within this sequence.

[c]Sequences are actual primer sequences (orientation 5'-to-3'). Sequences marked with an asterisk are complementary to the database sequences or the sequence given in Figure 1.--

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Please amend the paragraph beginning at line 26 of page 57 with the following rewritten paragraph:

--A cluster of EST sequences matching the genomic sequence of AL031290 were identified around nt 64000-66000 of AL031290, starting approximately 1.2 kb from the end of the PAPP-A2 encoding sequence. The existence of mRNA connecting the coding region of PAPP-A2 and this cluster was verified in a PCR using primers from AL031290 (5'-GGAAAGAGCAGAGTTCACCCAT-3' (SEQ ID NO:12), nt. 64900-64879 of AL031290) and the PAPP-A2 encoding sequence (5'-CCGTCTTAGTCCACTGCATCC-3' (SEQ ID NO:13), nt. 20499-20519 of AL031290, nt 5171-5191 of AF311940), and oligo-dT primed placental cDNA as a template (Overgaard et al., 1999, Biol Reprod 61, 1083-9). As expected, the size of the resulting product was 2.2 kb,

further demonstrating the existence of a PAPP-A2 mRNA with a 3'UTR of about 3 kb. The distribution among tissues is shown in Table 2.--

Please amend the paragraph beginning at line 30 of page 59 with the following rewritten paragraph:

--Construction of pPA2-KO: The construct pPA2-KO is a variant of the pPA2 expression construct in which residue Glu-734 of the active site of PAPP-A2 was substituted with a Gln residue. Thus, the mutant is E734Q. The pPA2-KO construct was made by site directed mutagenesis using the method of overlap extension PCR (Ho et al., 1989, Gene 77, 51-9) with pPA2 as the template. In brief, outer primers were 5'-CGCTCAGGGAAGGACAAGGG-3' (5' end primer, nt. 976-995 of SEQ ID NO:1) and 5'-CTAGAAGGCACAGTCGAGGC-3' (SEQ ID NO:14) (3' end primer, nt. 1040-1021, sequence of vector pcDNA3.1+). Overlapping internal primers were 5'-TGTCCCACTTGATGGATCATGGTGTCGGTGTGG-3' (SEQ ID NO:15) (nt. 2210-2178 of SEQ ID NO:1, nt. 2200 not C, but G resulting in E734Q) and 5'-CCATCAAGTGGGACATGTTCTGGGAC-3' (SEQ ID NO:16) (nt. 2196-2221 of SEQ ID NO:1, nt. 2200 not G, but C resulting in E734Q). The resulting mutated fragment was digested with *XbaI* and *XhoI* and swapped into pPA2 to generate pPA2-KO. All PCRs were carried out with *Pfu* DNA polymerase (Stratagene), and all constructs were verified by sequence analysis.--

Please amend the paragraph beginning at line 11 of page 60 with the following rewritten paragraph:

--Construction of pPA2-mH: Two primers (5'-GAGGGCCTGTGGACCCAGGAG-3', nt. 4906-4926 of SEQ ID NO:1, and 5'-GACGTAAAGCTTCTGATTTTCTTCTGCCTTGG-3' (SEQ ID NO:17), nt. 5373-5354 of SEQ ID NO:1, preceded by a HindIII site, AAGCTT, and nt. GACGTA to facilitate cleavage of the PCR product) were used in a PCR with pPA2 as the template to generate a nucleotide fragment encoding the C-terminal 156 residues of PAPP-A2 with the stop codon replaced by a *HindIII* site for in-frame ligation to expression vector. In brief, the PCR product was digested with *EcoRI* and *HindIII* and cloned into the *EcoRI/HindIII* sites of the vector pcDNA3.1/*Myc*-His(-)A to generate pPA2C-mH. The *NotI-XbaI* fragment (encoding the N-terminal portion of PAPP-A2), and the *XbaI-EcoRI* fragment (encoding the remaining central portion of PAPP-A2) were excised from pPA2 and ligated in one reaction into the *NotI/EcoRI* sites of pPA2C-mH. The resulting construct, pPA2-mH, encoded PAPP-A2 followed by residues KLGP (SEQ ID NO:18), the *myc* epitope (EQKLISEEDL (SEQ ID NO:19)), residues NSAVD (SEQ ID NO:20), and six H-residues (amino acids are given as one letter code). A stop codon follows immediately after the six histidine residues.--

Please amend the paragraph beginning at line 28 of page 61 with the following rewritten paragraph:

--C-terminally tagged PAPP-A2 purified from medium of cells transfected with construct pPA2-KO-mH (see examples 6.4 and 6.5) was reduced and run on a 10-20% SDS gel, and further blotted onto PVDF membrane (ProBlott, Applied Biosystems). Bands of 4 lanes were excised and subjected to N-terminal sequence analysis on an Applied Biosystems 477A sequencer equipped with an on-line HPLC (Sottrup-Jensen, 1995, Anal Biochem 225, 187-8). The N-terminal sequence observed at a level of approximately 20 pmol was: Ser-Pro-Pro-Glu-Glu-Ser-Asn (SPPEESN) (residues 234-240 of SEQ ID NO:2), resulting from cleavage before Ser-234 of the PAPP-A2 polypeptide after R(230)VKK (residues 230-233 of SEQ ID NO:2).--

Please amend the paragraph beginning at line 21 of page 62 with the following rewritten paragraph:

--For further analysis, recombinant IGFBP-5 was produced in mammalian cells. In brief, human placental oligo-dT primed cDNA (Overgaard et al., 1999, Biol Reprod 61, 1083-9) was used as a template to amplify cDNA encoding human IGFBP-5 (Accession number M65062). Specific primers containing an *XhoI* site (5'-TCCGCTCGAGATGGTGTTGCTCACCGCGGT-3' (SEQ ID NO:21)) and a *HindIII* site (5'-CGATAAGCTTCTCAACGTTGCTGCTGTCG-3' (SEQ ID NO:22)) were used,

and the resulting PCR product was digested and cloned into the *XhoI/HindIII* sites of pcDNA3.1/*Myc*-His(-)A (Invitrogen). The construct encoded the full-length proIGFBP-5, immediately followed by residues KLGP, the *myc* epitope (EQKLISEEDL (SEQ ID NO:19)), residues NSAVD (SEQ ID NO:20), and six H-residues (amino acids are given as one letter code). The construct was verified by sequence analysis. Plasmid DNA for transfection was prepared by QIAprep Spin Kit (Qiagen). Cell culture and expression of recombinant IGFBP-5 was performed as described above in Example 6.4.--

Please amend the paragraph beginning at line 22 of page 63 with the following rewritten paragraph:

--For cleavage site determination, purified rIGFBP-5 (Fig. 6, lane 7) was digested with purified PAPP-A2 and analyzed by SDS-PAGE (Fig. 6, lane 8). Edman degradation of blotted material showed that both distinct, visible degradation products (fig. 6, lane 8) contained the N-terminal sequence K(144)FVGGA (SEQ ID NO:23) (IGFBP-5 is numbered with the N-terminal Leu of the mature protein as residue 1). The two bands both represent intact C-terminal cleavage fragments, because they also contain the C-terminal c-myc tag (Fig. 6, lane 9); they are likely to be differently glycosylated, in accordance with the heterogeneity of purified rIGFBP-5 (Figure 6, lane 7). Both bands contained a second sequence at lower

level (45%), L(1)GXFVH (SEQ ID NO:24), corresponding to the N-terminal sequence of IGFBP-5. The absence of Ser, expected in the third cycle, was taken as evidence for carbohydrate substitution of Ser-3. O-linked glycan on the N-terminal cleavage fragment is likely to cause it to smear around the two distinct, C-terminal fragments. Sequence analysis on the reaction mixture (> 100 pmol) without SDS-PAGE separation showed only the same two IGFBP-5 sequences in equimolar amounts. Thus, PAPP-A2 cleaves IGFBP-5 at one site, between Ser-143 and Lys-144.--

IN THE SEQUENCE LISTING

Please substitute the attached Sequence Listing, numbered as pages 1-32 for the Sequence Listing previously submitted.

REMARKS

1. Applicants hereby submit the following:

[ ] a paper copy of a "Sequence Listing", complying with §1.821(c), to be incorporated into the specification as directed above;

[XX] an amendment to the paper copy of the "Sequence Listing" submitted on October 22, 2001, the amendment being in the form of substitute sheets;

[XX] the Sequence Listing in computer readable form, complying with §1.821(e) and §1.824, including, if an amendment to the paper copy is submitted, all previously submitted data with the amendment incorporated therein;

[ ] pursuant to §1.821(e), reference is made to the computer readable form filed on , in USSN , which presents the identical Sequence information, the use of which is now requested, in lieu of submitting a new computer readable form; and/or

[ ] a substitute computer readable form to replace one found to be damaged or unreadable.

[XX] 2. The description has been amended to comply with §1.821(d).

3. The undersigned attorney or agent hereby states as follows:

(a) this submission is not believed to include new matter [§1.821(g)];

(b) the contents of the paper copy (as amended, if applicable) and the computer readable form of the Sequence Listing, are believed to be the same [§1.821(f) and §1.825(b)];

(c) if the paper copy has been amended, the amendment is believed to be supported by the specification and is not believed to include new matter [§1.825(a)]; and

(d) if the computer readable form submitted herewith is a substitute for a form found upon receipt by the PTO to be damaged or unreadable, that the substitute data is believed to be identical to that originally filed [§1.825(d)].

4. Under U.S. rules, each sequence must be classified in <213> as an "Artificial Sequence", a sequence of

"Unknown" origin, or a sequence originating in a particular organism, identified by its scientific name.

Neither the rules nor the MPEP clarify the nature of the relationship which must exist between a listed sequence and an organism for that organism to be identified as the origin of the sequence under <213>.

Hence, counsel may choose to identify a listed sequence as associated with a particular organism even though that sequence does not occur in nature by itself in that organism (it may be, e.g., an epitopic fragment of a naturally occurring protein, or a cDNA of a naturally occurring mRNA, or even a substitution mutant of a naturally occurring sequence). Hence, the identification of an organism in <213> should not be construed as an admission that the sequence *per se* occurs in nature in said organism.

Similarly, designation of a sequence as "artificial" should not be construed as a representation that the sequence has no association with any organism. For example, a primer or probe may be designated as "artificial" even though it is necessarily complementary to some target sequence, which may occur in nature. Or an "artificial" sequence may be a substitution mutant of a natural sequence, or a chimera of two or more natural sequences, or a cDNA (i.e., intron-free

sequence) corresponding to an intron-containing gene, or otherwise a fragment of a natural sequence.

The Examiner should be able to judge the relationship of the enumerated sequences to natural sequences by giving full consideration to the specification, the art cited therein, any further art cited in an IDS, and the results of his or her sequence search against a database containing known natural sequences.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned "Version with markings to show changes made".

Respectfully submitted,

BROWDY AND NEIMARK
Attorneys for Applicant(s)

By: _____
Iver P. Cooper
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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the specification:

The paragraph beginning at line 31 of page 9 has been amended as follows:

Homology of PAPP-A2 with PAPP-A is evident upon alignment of the two amino acid sequences as shown in Figure 3. PAPP-A2 and PAPP-A share approximately 45% of their amino acid residues. Sequence motifs known to be important for the function of PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8; Lawrence et al., 1999, Proc Natl Acad Sci U S A 96, 3149-53; Overgaard et al., 2000, J Biol Chem) are also found in PAPP-A2. Principally, PAPP-A2 contains an elongated zinc binding motif (HEXXHXXGXXH (SEQ ID NO:3), amino acids shown by one letter code) at position 733-743 (Figure 2). This motif and a structurally important methionine residue, are strictly conserved within the metzincins, a superfamily of zinc peptidases (Bode et al., 1993, FEBS Lett 331, 134-40; Stocker et al., 1995, Protein Sci 4, 823-40).

The paragraph beginning at line 31 of page 50 has been amended as follows:

Figure 1 shows the cDNA sequence (in 5′→3′ orientation) corresponding to the mRNA that encodes preproPAPP-A2. Only the coding part of the sequence and the

terminal stop codon (*) is shown and is numbered 1-5376 of SEQ ID NO:1. The translated polypeptide sequence (SEQ ID NO:2) of preproPAPP-A2 is also shown. The signal peptide cleavage site was predicted using SignalP V2.0 to be after the alanine residue encoded by nt. 64-66 ((Nielsen et al., 1997, Protein Eng 10, 1-6), WWW prediction server is located at http://genome.cbs.dtu.dk/). The signal peptide of preproPAPP-A2 (nt. 1-66, 22 residues) is shown in bold. The nucleotide sequence of this figure represents nt. 1 to 5376 of SEQ ID NO:1. The protein sequence of this figure is illustrated as SEQ ID NO:2.

The paragraph beginning at line 26 of page 51 has been amended as follows:

Figure 3 shows the amino acid sequence of preproPAPP-A2 (SEQ ID NO:2) aligned with preproPAPP-A. The deduced amino acid sequence of preproPAPP-A2 (PA2) was aligned with the sequence of preproPAPP-A (PA) (SEQ ID NO:25) ((Haaning et al., 1996, Eur J Biochem 237, 159-63), AAC50543) using CLUSTAL W (Thompson et al., 1994, Nucleic Acids Res 22, 4673-80). Because the prepro-portion of PAPP-A did not show significant identity with the corresponding region of PAPP-A2, the alignment was manually adjusted to emphasize difference in length of pro-peptides. Arrows indicate the N-termini of the

mature proteins as found earlier for PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8) (Glu-81), and here for PAPP-A2 (Ser-234). Putative signal peptides, strongly predicted using SignalP V2.0 (Nielsen et al., 1997, Protein Eng 10, 1-6) are shown with lower case letters. The proportion of PAPP-A2 contains one other candidate initiation codon corresponding to Met-168, but no signal peptide was predicted following this residue using SignalP. The sequence motifs of PAPP-A (Kristensen et al., 1994, Biochemistry 33, 1592-8) are also found in PAPP-A2: The catalytic zinc binding motif and residues of the putative Met-turn are underlined and bolded in both sequences. Lin-notch motifs (LNR1-3) and short consensus repeats (SCR-1-5) are boxed. Cysteine residus are shaded. All cysteines of mature PAPP-A are also found in PAPP-A2. In addition, the secreted form of PAPP-A2 has four cysteine residues (Cys-343, Cys-533, Cys-618, and Cys-1268) with no counterpart in PAPP-A.

The paragraph beginning at line 8 of page 53 has been amended as follows:

Figure 7 shows the cDNA sequence of the PAPP-A2 mRNA coding region directly followed by the sequence of the 3'UTR. The sequence of the 3'UTR was obtained as detailed in Example 6.3. The first 5376 nucleotides of this sequence (nt. 1 -

5376) represents the coding sequence as illustrated in Figure 1 and SEQ ID NO:1 (nt. 1 - 5376). Nucleotides 5377 - 8527 of this sequence corresponds to the 3'UTR of the PAPP-A2 mRNA as illustrated in SEQ ID NO:~~3~~1 (nt. 5377 - 8527).

Table 1 beginning at line 20 of page 55 has been amended as follows:

TABLE 1. Locations of primers used for reverse transcription or PCR. The primers are listed in the order of their use.

NAME	SOURCE[a]	Nt. NUMBERS[b]	SEQUENCE[c]
RT-N-mid:	AL031290	10262-10281, (4770-4789)	GCTCACACACCACAGGAATG* (SEQ ID NO:4)
PR-mid5:	AL031734	141874-141894, (1947-1967)	GGCTGATGTGCGCAAGACCTG (SEQ ID NO:5)
PR-mid3:	AL031290	10208-10229, (4716-4737)	GCATTGTATCTTCAGGAGCTTG* (SEQ ID NO:6)
PR-N5:	AL031734	102606-102628, (-)	GAAGTTGACTTCTGGTTCTGTAG (SEQ ID NO:7)
PR-N3:	-	-, (2380-2400)	CCCTGGGAAGCGAGTGAAGCC* (SEQ ID NO:8)
RT-C:	AL031290	62982-63006, (-)	GCATTTCTTATAAGATCCTTCATGC* (SEQ ID NO:9)
PR-C5:	-	-, (4180-4201)	GACAGCTGTCCGTCATTGCTGC (SEQ ID NO:10)
PR-C3:	AL031290	62876-62897, (-)	CTTACTGCCTCTGAGGCAGTGG* (SEQ ID NO:11)

[a]Accession numbers of the relevant genomic clones are given. Primers PR-N3 and PR-C5 were located in the sequence connecting hom-N and hom-C, and are therefore not represented in the databases.

[b]Nucleotide numbers refer to the numbering of the sequences as reported in the file with the relevant accession number. In parentheses are given the corresponding numbers of SEQ ID NO:1 (Figure 1), except for primers PR-N5, RT-C and PR-C3, not within this sequence.

[c]Sequences are actual primer sequences (orientation 5'-to-3'). Sequences marked with an asterisk are complementary to the database sequences or the sequence given in Figure 1.

The paragraph beginning at line 26 of page 57 has been amended as follows:

A cluster of EST sequences matching the genomic sequence of AL031290 were identified around nt 64000-66000 of AL031290, starting approximately 1.2 kb from the end of the PAPP-A2 encoding sequence. The existence of mRNA connecting the coding region of PAPP-A2 and this cluster was verified in a PCR using primers from AL031290 (5'-GGAAAGAGCAGAGTTCACCCAT-3' (SEQ ID NO:12), nt. 64900-64879 of AL031290) and the PAPP-A2 encoding sequence (5'-CCGTCTTAGTCCACTGCATCC-3' (SEQ ID NO:13), nt. 20499-20519 of AL031290, nt 5171-5191 of AF311940), and oligo-dT primed placental cDNA as a template (Overgaard et al., 1999, Biol Reprod 61, 1083-9). As expected, the size of the resulting product was 2.2 kb, further demonstrating the existence of a PAPP-A2 mRNA with a 3'UTR of about 3 kb. The distribution among tissues is shown in Table 2.

The paragraph beginning at line 30 of page 59 has been amended as follows:

Construction of pPA2-KO: The construct pPA2-KO is a variant of the pPA2 expression construct in which residue Glu-734 of the active site of PAPP-A2 was substituted with a Gln

residue. Thus, the mutant is E734Q. The pPA2-KO construct was made by site directed mutagenesis using the method of overlap extension PCR (Ho et al., 1989, Gene 77, 51-9) with pPA2 as the template. In brief, outer primers were 5'-CGCTCAGGGAAGGACAAGGG-3' (5' end primer, nt. 976-995 of SEQ ID NO:1) and 5'-CTAGAAGGCACAGTCGAGGC-3' (SEQ ID NO:14) (3' end primer, nt. 1040-1021, sequence of vector pcDNA3.1+). Overlapping internal primers were 5'-TGTCCCACTTGATGGATCATGGTGTCGGTGTGG-3' (SEQ ID NO:15) (nt. 2210-2178 of SEQ ID NO:1, nt. 2200 not C, but G resulting in E734Q) and 5'-CCATCAAGTGGGACATGTTCTGGGAC-3' (SEQ ID NO:16) (nt. 2196-2221 of SEQ ID NO:1, nt. 2200 not G, but C resulting in E734Q). The resulting mutated fragment was digested with *XbaI* and *XhoI* and swapped into pPA2 to generate pPA2-KO. All PCRs were carried out with *Pfu* DNA polymerase (Stratagene), and all constructs were verified by sequence analysis.

The paragraph beginning at line 11 of page 60 has been amended as follows:

Construction of pPA2-mH: Two primers (5'-GAGGGCCTGTGGACCCAGGAG-3', nt. 4906-4926 of SEQ ID NO:1, and 5'-GACGTAAAGCTTCTGATTTTCTTCTGCCTTGG-3' (SEQ ID NO:17), nt. 5373-5354 of SEQ ID NO:1, preceded by a HindIII site, AAGCTT, and nt. GACGTA to facilitate cleavage of the PCR product)

were used in a PCR with pPA2 as the template to generate a nucleotide fragment encoding the C-terminal 156 residues of PAPP-A2 with the stop codon replaced by a *HindIII* site for in-frame ligation to expression vector. In brief, the PCR product was digested with *EcoRI* and *HindIII* and cloned into the *EcoRI/HindIII* sites of the vector pcDNA3.1/*Myc*-His(-)A to generate pPA2C-mH. The *NotI-XbaI* fragment (encoding the N-terminal portion of PAPP-A2), and the *XbaI-EcoRI* fragment (encoding the remaining central portion of PAPP-A2) were excised from pPA2 and ligated in one reaction into the *NotI/EcoRI* sites of pPA2C-mH. The resulting construct, pPA2-mH, encoded PAPP-A2 followed by residues KLGP (SEQ ID NO:18), the *myc* epitope (EQKLISEEDL (SEQ ID NO:19)), residues NSAVD (SEQ ID NO:20), and six H-residues (amino acids are given as one letter code). A stop codon follows immediately after the six histidine residues.

The paragraph beginning at line 28 of page 61 has been amended as follows:

C-terminally tagged PAPP-A2 purified from medium of cells transfected with construct pPA2-KO-mH (see examples 6.4 and 6.5) was reduced and run on a 10-20% SDS gel, and further blotted onto PVDF membrane (ProBlott, Applied Biosystems). Bands of 4 lanes were excised and subjected to N-terminal

sequence analysis on an Applied Biosystems 477A sequencer equipped with an on-line HPLC (Sottrup-Jensen, 1995, Anal Biochem 225, 187-8). The N-terminal sequence observed at a level of approximately 20 pmol was: Ser-Pro-Pro-Glu-Glu-Ser-Asn (SPPEESN) (residues 234-240 of SEQ ID NO:2), resulting from cleavage before Ser-234 of the PAPP-A2 polypeptide after R(230)VKK (residues 230-233 of SEQ ID NO:2).

The paragraph beginning at line 21 of page 62 has been amended as follows:

For further analysis, recombinant IGFBP-5 was produced in mammalian cells. In brief, human placental oligo-dT primed cDNA (Overgaard et al., 1999, Biol Reprod 61, 1083-9) was used as a template to amplify cDNA encoding human IGFBP-5 (Accession number M65062). Specific primers containing an *XhoI* site (5'-TCCGCTCGAGATGGTGTTGCTCACCGCGGT-3' (SEQ ID NO:21)) and a *HindIII* site (5'-CGATAAGCTTCTCAACGTTGCTGCTGTCG-3' (SEQ ID NO:22)) were used, and the resulting PCR product was digested and cloned into the *XhoI/HindIII* sites of pcDNA3.1/*Myc*-His(-)A (Invitrogen). The construct encoded the full-length proIGFBP-5, immediately followed by residues KLGP, the *myc* epitope (EQKLISEEDL (SEQ ID NO:19)), residues NSAVD (SEQ ID NO:20), and six H-residues (amino acids are given as one letter code). The construct was

verified by sequence analysis. Plasmid DNA for transfection was prepared by QIAprep Spin Kit (Qiagen). Cell culture and expression of recombinant IGFBP-5 was performed as described above in Example 6.4.

The paragraph beginning at line 22 of page 63 has been amended as follows:

For cleavage site determination, purified rIGFBP-5 (Fig. 6, lane 7) was digested with purified PAPP-A2 and analyzed by SDS-PAGE (Fig. 6, lane 8). Edman degradation of blotted material showed that both distinct, visible degradation products (fig. 6, lane 8) contained the N-terminal sequence K(144)FVGGA (SEQ ID NO:23) (IGFBP-5 is numbered with the N-terminal Leu of the mature protein as residue 1). The two bands both represent intact C-terminal cleavage fragments, because they also contain the C-terminal c-myc tag (Fig. 6, lane 9); they are likely to be differently glycosylated, in accordance with the heterogeneity of purified rIGFBP-5 (Figure 6, lane 7). Both bands contained a second sequence at lower level (45%), L(1)GXFVH (SEQ ID NO:24), corresponding to the N-terminal sequence of IGFBP-5. The absence of Ser, expected in the third cycle, was taken as evidence for carbohydrate substitution of Ser-3. O-linked glycan on the N-terminal cleavage fragment is likely to cause it to smear around the

two distinct, C-terminal fragments. Sequence analysis on the reaction mixture (> 100 pmol) without SDS-PAGE separation showed only the same two IGFBP-5 sequences in equimolar amounts. Thus, PAPP-A2 cleaves IGFBP-5 at one site, between Ser-143 and Lys-144.